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APPLICATION OF THE SECOND DERIVATIVES OF UV SPECTRA FOR THE IDENTIFICATION OF β-CASEIN HYDROLYSATE COMPONENTS

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The objective of the present study was to develop methods for the identification of β-casein and the components of its hydrolysate released by plasmin, on the basis of the second derivatives of UV spectra and the parameters describing them. These parameters characterise the location of the most common extrema of derivatives of UV spectra in proteins. β-Casein was hydrolysed by plasmin. The products obtained were isolated and separated by ultrafiltration and reversed-phase high-performance liquid chromatography (RP-HPLC) coupled with a photodiode detection system. In our experiment this detection method was used for a qualitative analysis of β-casein and components of its hydrolysate on the basis of parameters characterising UV spectra. The results obtained indicate the suitability of RP-HPLC and analysis of the second derivatives of UV spectra for the identification of proteins and peptides showing different concentrations of tyrosine and other aromatic amino acids.

INTRODUCTION

β-Casein (β-CN) constitutes about 35% of total casein in milk. This phosphoprotein, composed of 209 amino acid residues, has molecular mass of approx. 24 kDa [Eigel *et al.*, 1984; Imafidon *et al.*, 1997]. Its C-terminal segment is built of a strongly hydrophobic domain, containing most of total non-polar amino acid residues, whereas its N-terminal segment is strongly hydrophilic [Swaisgood, 1992]. A distinguishing feature of β-CN structure, resulting from a specific distribution of polar and hydrophobic amino acids, is its amphiphilic nature [Dickinson, 1999]. Due to a high content of proline residues (approx. 17%) [Swaisgood, 1992], uniformly distributed along the polypeptide chain, β-casein is characterised by a flexible structure [Darewicz *et al.*, 2000; Darewicz, 2001]. Holt and Sawyer [1993] proposed β-CN be assigned to the class of the so-called rheomorphic proteins. β-Casein is present in several genetic variants, differing in the amino acid sequence and/or the number of added phosphate residues. The most common genetic variants of β-CN are: A1, A2, A3, B, C, D, E, F, G [Dong Ng-Kwai-Hang, 1998; Swaisgood, 1992; Visser *et al.*, 1995], and H [Han *et al.*, 2000].

Milk contains about 60 native enzymes [Fox Stepaniak, 1993]. The major native proteolytic enzyme found in milk is plasmin (EC 3.4.21.7), a serine proteinase obtained from the blood plasma. Plasmin shows its optimum activity at pH 7.4–7.5 and a temperature of 37°C [Bastian Brown, 1996]. Plasmin hydrolyses bonds formed with lysine (K-X) and arginine (R-X), with a marked preference for the former [Bastian *et al.*, 1991]. The products of β-casein hydrolysis by plasmin are the following γ-caseins: γ_1-CN (fraction 29–209), γ_2-CN (f 106–209), γ_3-CN (f 108–209), γ_4-CN (f 114–209); and proteose-peptones: PP5 (f 1–105/107; 5P), PP8S (f 29-105/107; 1P) and PP8F (f 1–28; 4P) [Darewicz *et al.*, 2000; Eigel *et al.*, 1984; Fox Stepaniak, 1993; Mc Sweeney, 1997]. The presence of the β-casein fragment (f 29–105/107) may indicate proteolytic changes, especially in milk obtained from cows with subclinical mastitis [Le Roux *et al.*, 1995; Mc Sweeney, 1997].

Mass spectrometry is one of the most effective methods for protein and peptide identification. However, its is also very expensive. A relatively inexpensive alternative to mass spectrometry is the identification of proteins and peptides on the basis of their UV spectra obtained using a photodiode detector. Photodiode detection enables the identification of phenylalanine, tyrosine and tryptophan in proteins and peptides. UV spectroscopy can be coupled with HPLC [Perrin *et al.*, 1995; Zhao *et al.*, 1996]. The simplest method of interpretation of the spectra obtained with a photodiode detector is to calculate the absorbance ratio at various wavelengths [Lagerwerf, 1997; Wu *et al.*, 1986]. According to Bartolomè *et al.* [1997], the spectra of proteins and peptides can be identified on the basis of locations of maxima and minima in spectra and their first derivatives. However, maxima and minima appear in protein spectra at the same wavelengths, hence this method is not always reliable. The method developed by Perrin *et al.* [1995] for determining the ratios between aromatic amino acid concentrations on the basis of the first and second derivatives of spectra can be employed while calculating the ratios of aromatic amino acid concentrations in oligopeptides. In the case of longer polypeptide chains, the observed ratios between aromatic acid concentrations may differ from the actual ones [Darewicz *et al.*, 1999]. As reported by Dziuba *et al.* [2001], at low protein concentrations the minimum corresponding to the absorbance of Phe, indispensable for determining the Trp/Phe or Tyr/Phe ratio, disappears completely. The disap-

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pearance of this minimum may lead to overestimation of the above ratios, or make their calculation impossible. In recent years bovine milk proteins have been identified using the second and fourth derivatives of UV spectra, obtained by high-performance liquid chromatography (HPLC) coupled with photodiode detection [Dziuba *et al.*, 2001; Minkiewicz *et al.*, 2003].

Due to the fact that professional literature on the topic provides scant information on the methods of protein/peptide identification using UV spectroscopy, the objective of the present study was to determine the possibility of identifying β-casein hydrolysate components released by plasmin on the basis of new, more reliable attributes characterising the second derivatives of their UV spectra. In the present experiment reversed-phase high-performance liquid chromatography (RP-HPLC) was used for the separation of β-CN hydrolysate components, and UV spectroscopy – for their identification.

MATERIAL AND METHODS

Bovine β-casein was purchased from Eurial (Rennes, France), plasmin [EC 3.4.21.7] and aprotinin – from Sigma, and HPLC reagents – from Baker (the Netherlands).

Conditions of hydrolysis. β-Casein was hydrolysed by plasmin [Darewicz, 2001]. The reaction parameters were as follows: 2.5 mg/mL β-CN; enzyme/substrate = 1/2400 w/w; pH 6.8; temp. 40°C; time 5 h. The precipitate, which was forming constantly during hydrolysis, disappeared at a temperature lower than 4°C. Starting from the second hour of hydrolysis, the precipitate was separated by centrifugation (1500xg, 10 min, 30°C). Centrifugation was repeated every 0.5 h, and the precipitates obtained were put together and lyophilised. The reaction was inhibited by adding aprotitin to the hydrolysate (1/200; v/v). The supernatant obtained was ultrafiltered (MWCO 5 kDa). The components of β-casein hydrolysate, *i.e.* supernatant, retentate and permeate, were lyophilised.

Reversed-phase high-performance liquid chromatography (RP-HPLC). The chromatographic analysis was performed using a HPLC system (Shimadzu, Kyoto). The system consisted of two LC-10 AD pumps, a CTO 10-AS column oven, an N SCL-10 Advp controller, and a Jupiter C18 column 250 x 4.6 mm (Phenomenex). The program CLASS-VP ver. 5.3 (Shimadzu) was used for data collection and processing. Solvents A and B were mixtures of acetonitrile, water and trifluoroacetic acid (TFA) at a ratio of: 100:900:1 (v/v/v) and 900:100:0.7 (v/v/v), respectively [Visser *et al.*, 1991]. The following acetonitrile gradient was used: start 30% B, 43% B after 40 min, 80% B after 48 min, 30% B after 50 min. After gradient completion the column was equilibrated for 10 min. Separation was performed at 30°C. The flow rate was 0.8 mL/min, and injection volume – 50 μL. Samples of β-casein and components of its hydrolysate were prepared by dissolving 3.0 mg of lyophilisate in 1 mL of buffer A with 6 mol/L of urea. The UV spectra were recorded in a 190–300 nm wavelength range.

Mass spectrometry (MALDI-TOF-MS). Mass spectrometry with ionisation by desorption in the matrix was performed according to the method described by Darewicz [2001]. Mass spectra were obtained using a mass spectrometer Voyager-De™ RP (PerSeptive Biosystem, USA), at accelerating voltage 25 kV and pressure approx. 2.1×10^{-7} torr. The matrix solution was obtained by dissolving 10 μg of sinapic acid in 1 L of a mixture of acetonitrile, 0.3% TFA and deionised water (3:1:6, v/v/v). One μL of the sample mixed with 9 μL of the matrix solution were loaded on the welled plate. The laser emitted impulses at a wavelength of 337 nm, generating 2040 impulses per second. All samples were transferred in three replications.

Parameters of protein/hydrolysate component identification. The spectra were described using the parameters A, B and C, characterising the wavelengths at which the minima and maxima of the second derivatives of UV spectra of proteins occur most often.

$$A = [(d^2 A/d\lambda^2)_{280} - (d^2 A/\lambda^2)_{283}] / A_{220}$$
$$B = [(d^2 A/d\lambda^2)_{280} - (d^2 A/\lambda^2)_{283}] / A_{240}$$
$$C = A_{280} / A_{220};$$

where A – absorbance; λ – wavelength.

Average wavelengths corresponding to particular minima and maxima are given in the above formulas. They vary within a range of 2 nm around the average values. In the case of the lack of the maximum and minimum within the above range the derivative value at a given wavelength was used for calculations [Dziuba *et al.*, 2001]. The statistical calculations were done using STATISTICA PL software. The significance of differences between two means was determined by the t-test for independent samples, at significance levels of α=0.001; 0.01; 0.02 and 0.05.

RESULTS AND DISCUSSION

Hydrolysis of β-casein by plasmin was constantly monitored by chromatographic separation (RP-HPLC) of the hydrolysate obtained, and analysis of its mass spectrum. The acetonitrile gradient used in the experiment enabled the separation of two genetic variants of β-casein by RP--HPLC (Figure 1). The MALDI-TOF analysis of mass spectra showed that β-casein used in the study consisted of two genetic variants: A1 and A2. Under conditions of proteolysis of β-casein by plasmin it was possible to obtain the following hydrolysate components: precipitate, supernatant, retentate and permeate. The identification of particular peptide fractions of β-CN, obtained using plasmin, was based on the molecular masses of peptide segments, determined by MALDI-TOF-MS, the amino acid sequence in the polypeptide chain of β-casein, and specificity of plasmin effects. Mass spectrometry is currently the most accurate method of protein and peptide identification on the basis of their molecular masses. This method allows measurements of the molecular mass of a given substance exact to 0.02–0.05% [De Hoffman, 1998]. Such precision is sufficient to distinguish between peptides with molecular masses of over 3 kDa, containing an oxidised or reduced methionine residue [Minkiewicz *et al.*, 1996]. The MALDI-TOF analysis of mass spectra showed that the precipitate contained the β-CN fragments (f 106/108/114–209) and low--molecule peptides from the C-terminal segment of β-casein

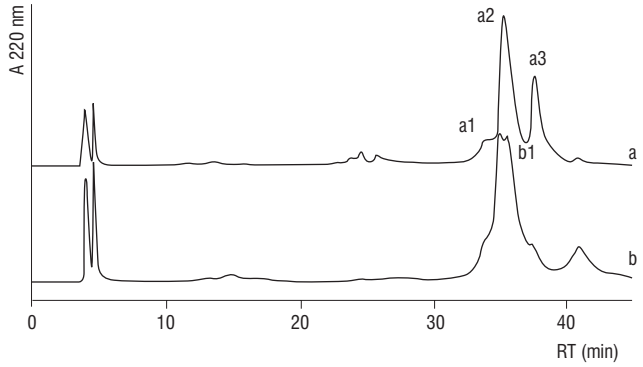

FIGURE 1. Chromatograms: (a) precipitate formed as a result of β-casein hydrolysis by plasmin, and (b) β-casein A^1 A^2 (a1, a2, a3, b1 – fractions whose second derivatives of spectra are shown in Fig. 2). The separations were performed under the following conditions: Jupiter C18 column; temp. 30°C; TFA concentration in solvent B – 0.7 mL/L; acetonitrile gradient – start 30% B, 43% B after 40 min, 80% B after 48 min, 30% B after 50 min; β-casein concentration – 3 mg/mL; flow rate – 0.8 mL/min; injection volume – 50 μL.

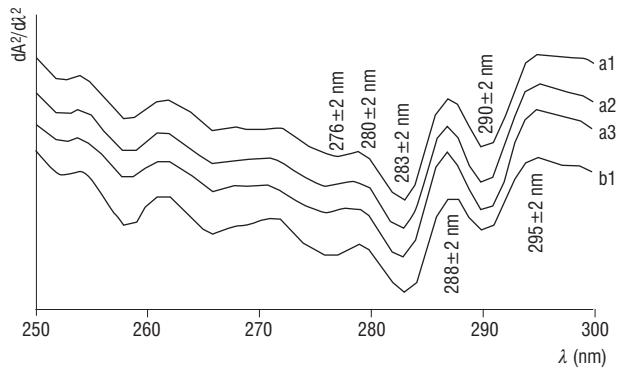

FIGURE 2. Second derivatives of UV spectra of β-casein fragments present in the precipitate (a1, a2, a3, Fig. 1), and β-casein (b1, Fig. 1). The spectra were obtained analyzing the chromatograms shown in Fig. 1.

(*e.g.* f 177–209), the retentate contained the following fragments: (f 1–105/107), (f 29–105/107) and peptides from the C-terminal segment of β-CN (*e.g.* f 114–169; f 108–169), and the supernatant contained the fragments (f 1–28), (f 170–176) and (f 106–113), in addition to those detected in the retentate.

The chromatographic analysis of the products of β-casein proteolysis indicated that their retention times were similar to or shorter than the retention time of the native protein. The precipitate was flowing out of the column more or less simultaneously with β-casein (Figure 1). The amino acid sequence of β-CN [SWISS-PROT database] shows that most residues of hydrophobic amino acids can be found in the C-terminal segment of its protein chain. Mean hydrophobicity of the C-terminal segments contained in the precipitate is higher than in the whole polypeptide chain of β-casein. According to reference data [de Collonque *et al.*, 1994; Wilce *et al.*, 1995], an increase in the hydrophobicity of amino acids forming a given protein/peptide, and an increase in the molecular masses of proteins/peptides, contribute to an increase in retention time. In the case of β-CN and precipitate, the opposite effects of these two elements of molecular characteristics resulted in similar retention times. The supernatant, permeate and retentate, containing hydrophilic and amphiphilic fragments of β-CN, were characterised by shorter retention times than the native protein.

Spectrum fragments in the 270–300 nm range can provide a basis for the identification of β-casein and the components of its hydrolysate. UV spectra of proteins are similar to one another. The similarity indices (cosine of the angle between spectra presented as vectors in a multidimensional space) of β-CN and the components of its hydrolysate at a wavelength of 270 to 300 nm assumed values higher than 0.99. The differences between β-CN and the components of its hydrolysate may be made more visible using the second derivatives of their spectra. Figure 2 shows the second derivatives of UV spectra of β-casein and its fragments present in the precipitate. The wavelengths at which it is possible to measure the second derivatives of spectra used for protein identification [Dziuba *et al.*, 2001] are also given in this Figure. The minimum depth of the second derivative applied while determining the parameters A and B is directly proportional to tyrosine content [Palladino *et al.*, 1991; Zhao *et al.*, 1996]. The absorbance value at a wavelength of 280 nm, used for calculating the parameter C, depends on the concentrations of aromatic amino acids [Moffat *et al.*, 2000]. At a wavelength of 220 or 240 nm, absorbance is proportional to the number of peptide bonds, although the maximum absorbance of these bonds is observed at a wavelength of approx. 210 nm [Lottspeich *et al.*, 1984].

Table 1 presents the values of parameters A, B and C, as well as the standard deviations for β-casein and the components of its hydrolysate: precipitate, supernatant, retentate and permeate. The values of the parameters characterizing the UV spectra of all three precipitate fractions (Figure 1) were the same. The values of parameters A, B, C for the precipitate were higher than their values determined for non-hydrolysed protein and the other components of β-CN hydrolysate. The MALDI-TOF-MS analysis showed that the major components of the precipitate were the β-casein fragments 106/108/114–209. The proportion of aromatic amino acids in these fragments is higher than in the native protein, which was confirmed by the observed values of parameters A, B and C. In the case of retentate lower values of spectroscopic parameters, as compared with the values determined for supernatant, indicate lower total concentration of aromatic amino acids in peptides present in

TABLE 1. Values of the parameters A, B and C of UV spectra of β-casein and the components of its hydrolysate released by plasmin.

Protein/ hydrolysate component	A±SD*	B±SD*	C±SD*
β-Casein A^1A^2	0.00028±0.00002 n=9	0.00347±0.00026 n=9	0.04839±0.00068 n=9
Precipitate	0.00036±0.00002 n=9	0.00492±0.00039 n=9	0.05849±0.00138 n=9
Supernatant	0.00035±0.00003 n=9	0.00531±0.00081 n=9	0.04681±0.00031 n=9
Permeate	0.00005 ±0.00001	0.00030 ±0.00008	0.02190±0.00170 n=9
Retentate	0.00026±0.00002 n=12	0.00380±0.00039 n=12	0.01967±0.00149 n=12

* mean values of the parameters determined for β-casein, precipitate, supernatant, permeate and retentate.

TABLE 2. Statistical significance of differences between the parameters A, B and C, determined for β-casein and the components of its hydrolysate released by plasmin.

Protein/hydrolysate component	β-casein	Precipitate	Supernatant	Permeate	Retentate
β-Casein	---	A**** B****C****	A*** B**C****	A**** B****C****	A** B*C****
Precipitate	A**** B****C****	---	A*** B**C***	A**** B**** C****	A**** B****C****
Supernatant	A*** B**C****	A*** B**C***	---	A**** B**** C****	A*** B**C**
Permeate	A**** B**** C****	A**** B**** C****	A**** B**** C****	---	A**** C***
Retentate	A** B*C****	A**** B****C****	A*** B**C**	A**** C***	---

Statistically significant differences at: *p<0.05; **p<0.02; ***p<0.01; ****p<0.001.

this component of β-CN hydrolysate, or a higher value of mean length of their polypeptide chains. These findings are consistent with the results of identification of peptides contained in these hydrolysate components, performed by mass spectrometry. The β-casein fragment 1–28 dominated in the permeate. Only the presence of additional fragments, formed as a result of hydrolysis by plasmin, *i.e.* (f 170–176) and (f 106–113), allowed determining the parameters A and B (based on tyrosine content) and parameter C. Table 2 presents statistical significance of differences between the parameters A, B and C of UV spectra of β-casein and the components of its hydrolysate released by plasmin. In most cases in the majority of pairs (protein/hydrolysate component or two hydrolysate components) there were statistically significant differences at a level of $p<0.001$ between the parameters A, B and C, *e.g.* for the pair precipitate/retentate. In the other cases significant differences were found at a level of $p<0.02$ and 0.01. Only in the case of parameter B, used for differentiating between β-casein and retentate, the difference was statistically significant at $p<0.05$.

CONCLUSIONS

1. The conditions of β-casein hydrolysis applied in the experiment enabled obtaining hydrolysate components being the source of peptides with different molecular characteristics, *i.e.* hydrophilic, amphilphilic and hydrophobic.

2. The retention times of hydrolysate components released from β-casein by plasmin, measured by reversed-phase high-performance liquid chromatography, were increasing with an increase in the hydrophobicity of amino acids contained in peptide sequences, and with an increase in molecular mass.

3. Retention times are not sufficient for the identification of particular components of plasmin-hydrolysed β-casein.

4. Analysis of the second derivatives of UV spectra enables the identification of β-casein and the components of its plasmin hydrolysate. Identification can be based on the parameters describing UV spectra, depending on the concentrations of aromatic amino acids and number of peptide bonds.

5. Parameters A and C can be recommended for the identification of β-casein and the components of its plasmin hydrolysate.

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WYKORZYSTANIE DRUGICH POCHODNYCH WIDM UV DO IDENTYFIKACJI SKŁADNIKÓW HYDROLIZATU KAZEINY-β

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Celem pracy było opracowanie metod identyfikacji kazeiny-β i składników hydrolizatu uwolnionych przez plazminę na podstawie drugich pochodnych widm UV i parametrów je opisujących. Parametry te charakteryzują położenie najczęściej występujących ekstremów pochodnych widm UV białek. Kazeinę-β poddano hydrolizie przez plazminę. Powstałe produkty wyizolowano i rozdzielono z zastosowaniem ultrafiltracji oraz wysokosprawnej chromatografii cieczowej w odwróconej fazie (RP-HPLC), skojarzonej z fotodiodowym systemem detekcji. Przedstawiono zastosowanie tego sposobu detekcji do analizy jakościowej kazeiny-β i składników jej hydrolizatu na podstawie opracowanych parametrów charakteryzujących widma. Uzyskane wyniki świadczą o przydatności metody RP-HLPC oraz analizy drugich pochodnych widm UV do identyfikacji białek i peptydów różniących się zawartością tyrozyny i pozostałych aminokwasów aromatycznych.